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« Growth arrest-specific gene 6 » (Gas6) as an intra-hospital mortality predictor for patients in septic shock

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N.B.: A la fin du mois de novembre, cinquante nouveaux cas ont été rajouté à notre étude. De ce fait, nous ne pouvons pas remettre à la Faculté la version finale de l'article dans les temps impartis par le Travail de Master.

Ainsi, comme convenu avec la Professeur A. Angelillo-Scherrer, une version intermédiaire de l'article vous est transmis, comprenant un descriptif de mon implication dans cette étude.

Dans le cadre de ce travail fait conjointement avec un médecin doctorant, mon rôle a consisté en:

- récolte et tri des échantillons sanguins
- aide au travail de laboratoire (préparation et analyse)
- recherche, tri et établissement d'une base de données concernant les patients inclus dans l'étude
- m'occuper de toute la partie épidémiologique et populationnelle de l'étude
- analyse statistique
- participation à l'écriture de l'article
- écriture d'un poster et présentation orale à la "poster session" au Congrès Suisse de Médecine Interne en mai 2011
- présentation de nos résultats au colloque du Service d'hématologie (à venir)

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Article intermédiaire :

« Growth arrest-specific gene 6 » (Gas6) as an intra-hospital mortality predictor for patients in septic shock

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Abstract

Aim: Gas6 is known to be elevated in sepsis, correlating with the severity of infection and organ failure. We aimed to investigate the performance of Gas6 plasma levels at admission to predict the risk of mortality in a cohort of septic patients.

Methods: We used prospectively collected data and plasma samples from the “Sepsis Cohorte Romande”. Gas6 level was measured by ELISA at admission and expressed in percentage relative to its level in a pool of normal plasma.

Results: Non-survivors (n=21) presented higher Gas6 levels than survivors (n=73) (median 258% vs 164%, IQR 194 and 117 respectively) (p=0.0027). Gas6 correlated positively with different cytokines and was the best mortality predictor, as shown by the ROC curves area. In patients with septic shock (n=66), using 249% as a cut-off value, Gas6 measurement had a specificity of 67% and a sensitivity of 81% for predicting mortality. ROC curve area was 0.75. Positive and negative predictive values were 57% and 87%, respectively.

Conclusion: Thus, Gas6 plasma level at admission might be a useful tool to predict mortality in patients with septic shock. Although Gas6 hold promise as an early sepsis marker, its precise implication in sepsis remains to be elucidated. Our observation should be further investigated in larger prospective clinical trials.

Introduction

Sepsis is the presence of a systemic inflammatory response syndrome (defined by two or more of fever or hypothermia, tachypnea, tachycardia, leukocytosis or leukopenia or >10% bands) with a proven or suspected microbial aetiology. It is referred as severe when signs of organ dysfunction occur and as septic shock when sepsis-induced hypotension ensues or when vasopressors or inotropes are necessary to maintain blood pressure despite adequate fluid resuscitation, in the presence of perfusion abnormalities(1,2).

Annual incidence of severe sepsis in United States has been estimated to 3 cases per 1000 population with a mortality rate of 28,6%(3). In Europeans Intensive Care Units (ICU), frequency of sepsis has be reported to be as high as 35% with a mortality of 27% and 50% for patients in sepsis and septic shock, respectively(4). Gram-positive bacterias are the leading cause of sepsis, followed by Gram-negative and fungal infection.

The pathophysiology of sepsis is complex(5). It can be seen as an inappropriate immune response to pathogen invasion, which results from both hyper-inflammatory state and immunoparalysis. On one hand, the activation of immune cells and endothelium after exposition to microbial products leads to cytokine production and coagulation activation. This further activates immune cells, which give rise to a second burst of cytokine resulting in amplification of the inflammatory response. This « cytokine storm » as well as coagulation activation result in microvasculature trouble and in increased vascular dilatation and permeability which cause tissue ischemia and organ failure(6). This classical conception of sepsis pathophysiology is now believed to represent only the early phase of this process and would be in fact responsible of a minor proportion of mortality. On the other hand, anti-inflammatory molecules are also secreted in an attempt to controlate immune reaction during sepsis. Furthermore, apoptosis induced by cytokines such as TNF-alpha results in down-regulation of inflammation: lymphocytes and dendritic cells death and macrophage phagocytosis of apoptotic cells result in loss of immune effector cells and to further anti-inflammatory molecules secretion(7). This compensatory anti-inflammatory response syndrome may results in immunoparalysis with inability to clear the original pathogen invasion or predisposes to secondary infection(8).

No biomarker has been found to accurately diagnose and monitor septic patients. Clinical score based on physiologic measurement and laboratory parameters, such as APACHE or

SOFA scores, are the currently tools used to follow patient's evolution. Mortality in sepsis is more or less stable over the paste decade. To date, the only proved adjunctive drug treatment which reduces it in patients with severe sepsis or septic shock is recombinant activated Protein C(9).

Gas6, whose gene was first described in fibroblast during growth arrest phase(10), is a vitamin K-dependent secreted protein also expressed in endothelial(11), vascular smooth muscle(12), bone marrow(13) and central nervous cells(14). It shares 44% homology with the amino acid sequence of Protein S, a coagulation regulatory protein which acts as a Protein C co-factor(11). Gas6 is composed of four domain: a Gla domain, which require gamma-carboxylatation to be functional and interacts with membrane phospholipids, four EGF-like domains and a Sex-Hormone Binding like domain which interacts with its receptors Tyro-3, Axl, Mer(15). Those form the TAM family and are tyrosine kinase exhibiting a widespread distribution in adult tissues (16). Gas6 bind the three receptors with differing potency (Axl>Mer>>>Tyro3)(15) while Protein S activates Tyro3(17) and Mer(18). Recently, two additional TAM family ligand were found: tubby and tubby-like protein (19). Gas6 and its receptors are implicated in a number of cellular functions including reversible growth arrest(10), survival(20), proliferation(12,20), adhesion(21) and migration(22).

They have been found to participate in a number of pathophysiological events linked to sepsis (Fig. 1). Gas6 is a regulator of innate immunity(23,24), promoting an anti-inflammatory comportment in antigen-presenting cells by inhibition of cytokine production(23,25) and facilitation of apoptotic cells phagocytosis(26). However, it acts on an opposite way on endothelium as more adhesion molecule and cytokine secretion occur after its activation by Gas6(27). Murine sepsis models with Gas6 or TAM KO mice have thus been developed (28)(L. Burnier, unpublished data): LPS injection, intraperitoneally E. coli inoculation or caecal ligation and puncture lead to higher inflammatory cytokines levels and death in these mice, indicating that Gas6 may have a protective role during sepsis.

In human, plasma Gas6 level is known to be elevated in inflammatory conditions, including sepsis(29)and correlates with organ dysfunction and disease severity(30). Four studies have examined its plasmatic levels in patients with sepsis(29-32) without finding differences between survivors and non-survivors at admission. It should be emphasized

that three of them disposed of a limited septic patients number (<50)(30-32) and one comprised only 5% of non-survivors (8 on 169 sepsis cases)(29).

As we disposed of a cohort of septic patients of a certain importance with a more representative non-survivors number, we aimed to prospectively investigate the performance of Gas6 plasma levels at admission to predict the risk of mortality.

Material and methods

Subjects

The study cohort is composed of 113 patients from «Sepsis Cohorte Romande» (SEPCORO). Briefly, adults subjects presenting severe sepsis or septic shock, according to the standards definitions(2,33), were prospectively enrolled in Intensive Care Unit (ICU) of surgery or medicine, in Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland, from February 2008 to august 2010. Blood sampling were performed at admission (J0), J3, J7 and then weekly until discharge from ICU or death and conserved at -80°C until use. Exclusion criterias were HIV status, haematological malignancy or immunosuppressive treatment.

The research protocol was accepted by the research ethical committee of the faculty of Biology and Medicine, Lausanne University, Switzerland.

Gas6 and sAxl ELISAs

Plates (96 wells Maxisorp™, Nunc) were coated with 100 µL per well of polyclonal goat anti-human Gas6 antibody (AB885, R&D Systems) diluted in 0.1M $NaHCO_3$ pH 8.2 and incubated overnight at 4°C. After two washing with PBS-Tween 0.05%, they were blocked with 100 µL PBS-BSA 1%- sucrose 5% and then incubated 2 hours at room temperature. Samples diluted 50 and 100 times and normal plasma serial dilution with PBS-BSA 1% were added after three additional washes, followed by an overnight incubation at 4°C. After three washing, we used biotynitilated polyclonal goat antibody (BAF885, R&D Systems) for the detection, adding 100 µL in each well, and leaving it two hour at room temperature. Signal was then amplified with Avidin-HRP (BD Pharmingen™) and plates incubated during 20 minutes at 37°C. Finally, OPD (SIGMA-ALDRICH®) was added and plates were blocked with 50 µL HCL 3M 5 minutes later. Absorbance was

measured at 492 nm and the result was expressed in percentage relative to normal plasma, using its serial dilution as standard curve.

We used a commercial kit from R&D Systems (DY154) to measure sAxl, following the instruction provided.

Statistical analysis

Nonparametric tests were used throughout the study. We used the two-sample Wilcoxon rank-sum (Mann-Whitney) test to evaluate differences between groups and Spearman's rank correlation coefficient for correlations.

To study Gas6 evolution in time, we used a linear mixed model, calculated with aleatory constants and effects. These were employed for the estimation of the effect of survival, time and interaction between time and survival on Gas6. A non-structured covariance between the repeated measures was used.

The relation between Gas6 and the other variable measured was assessed with univariate linear regression. Then, univariate logistic regression was performed to examine the association between the mortality and each of the predictor separately. Taking into account the number of non-survivors and the fact that the outcome (survival or non-survival) was binary, we used univariate logistic regression to test the independence of Gas6 effect on mortality versus each confondant found with the method described above..

Receiver operating characteristic (ROC) curve was employed to examine the performance of variables to predict intra-hospital mortality and Kaplan-Meier curves were drawn to study survival.

Statistical analyses and graphics were performed using the softwares STATA and Prism Graphpad.

Results

Study population

94 patients were finally enrolled comprising 73 survivors (77.7%), a proportion of 57 males (60.6%) and a median age of 65 years old (P25-P75 : 50-74)(Table 1). We reported 66 septic shock (70.2%) and 28 severe sepsis (29.8%). The gravity scores used were Acute Physiology and Chronic Health Evaluation II (APACHE II) with median value 27.5

points (P25-P75: 23.3-37) and Sequential Organ Failure Assessment (SOFA) with median value 11 points (P25-P75: 9-13). The median value for the c-reactive protein (CRP) was 231.5 mg/L (P25-P75 : 130.8-315) and for the procalcitonin (PCT) 22.3 µg/L (P25-P75 : 5.2-53.3). The three main diagnosis for ICU (Intensive Care Unit) admission were pneumonia (33%), peritonitis (26.6%), fasciitis (12.8%) and most common pathogens were *E. Coli* (23.4%), *S. Pneumoniae* (19.1%), *S. pyogenes* (10.6%) ; we have to notice that in 13.8% no pathogen was found.

Plasma levels of Gas6

Gas6 plasma levels at admission were higher in patients with fatal outcome (median 258%, IQR 194) than in survivors (median 164%, IQR 117) (n=94, p=0.0027)(Fig. 2). As blood sample were performed in different number at different time points, we decided to make a model to give us a mean to analyse repeated measures. The linear mixed model to study Gas6 evolution during ICU stay showed that, all time confound, survivors had a mean level of Gas6 96.6 units lower than non-survivors. As the interaction between survival and time was not statistically significant, suggesting that difference in Gas6 level between survivors and non-survivors remained constant in time, we made an other model (Fig. 3). With this one, non-survivors exhibited a mean Gas6 level of 255.9% versus 176.1% in survivors at admission. It then diminished from 0.66 units each day in both groups, however, this diminution was not statistically significant. Whatever model we used, Gas6 curve remained more elevated in non-survivors than in survivors.
As the number of non-survivors diminished rapidly after J0 to become statistically not significant, we focused our next analysis on results from the first blood sample.

Correlations of Gas6 with clinical scores and markers of inflammation

Cytokines were measured in 90 out of the 94 patients of our cohort. Gas6 at admission correlated positively with plasma levels of IL-6, IL-8 and IL-10 (Table 2). Correlations with IL-1b and TNF-alpha were not significant. There were no correlation between Gas6 and CRP, procalcitonin, SOFA and APACHEII scores.
IL-1beta, IL-6, IL-8, IL-10, TNF-alpha and PCT were tested to find if they were in relation with Gas6. Univariate linear regression showed that IL-10 was the only marker to be in relation with Gas6 but, according to univariate logistic regression, it did not influence

mortality significatively (p=0.176). Furthermore, multivariate logistic regression showed that Gas6 effect on mortality was independent from IL-10.

In fact, Gas6 admission level predicted mortality better than the other tested markers, as shown by the ROC curves areas (Table 2).

Gas6 level at admission in ICU can predict intra-hospital mortality in patient with septic shock

Septic shock is known to have poorer outcome than severe sepsis, as illustrated by the number of patients with fatal outcome in each group of our cohort (n=20 vs 2). Thus, we decided to focus our survival analyses on this population.

In patients with septic shock (n=66), considering 249% as a cut-off value, Gas6 measurement had a specificity of 66.7% (95% CI: 41%-86.7%) and a sensitivity of 81.3% (95% CI: 67.4%-91.1%) with a positive likelihood ratio of 2.44 for predicting mortality. ROC curve area was 0.75 (SE: 0.07, 95% CI: 0.60-0.89)(Fig. 4). Positive and negative predictive values for mortality were 57% and 87%, respectively. Kaplan-Meier curves comparison showed that there was a significant difference (p=0.0009) between survival times in the two groups (Fig. 5).

sAxl level at ICU admission also discriminate between survivors and non-survivors

Gas6 has been found to circulate in human plasma bound to one of its receptor in soluble form, sAxl(34). Thus, we decided to measure sAxl in the ICU admission blood sample. Survivors exhibited lower sAxl plasma levels than non-survivors (median 34 ng/ml vs 47 ng/ml, IQR 15.5 and 25.5 respectively) (p=0.01)(Fig. 6). In the entire population, sAxl correlated positively with Gas6 and TNF-alpha (rho 0.35 and 0.24;n=94 and 90 respectively; $p<0.05$). ROC curve area for sAxl was 0.69 in the entire population and 0.72 in septic shock group.

Discussion

Opposite to our hypothesis based on observations of murine sepsis models, non-survivors exhibit higher Gas6 plasmatic level than survivor. Indeed, the role of Gas6 in sepsis is not so clear even in murine models, as highlighted by the fact that its adjunction

in Gas6 KO mice does not lead to any survival improvement (L. Burnier et al. unpublished data).

Gas6 is described as a immune immunity modulator. When binding the TAM receptors, it downregulates dendritic cells cytokine production induced by Toll-like receptor (TLR) activation, through the TLR suppressors SOCS1 and SOCS3(23). Alciato et al. have found that TNF-alpha, IL-6 and IL-1 expression in monocytes/macrophages was inhibited by Gas6 with a pathway involving PI3K/Akt/GSK3beta and repression of NF-κB(25). Phosphatidylserines, phospholipids normally confined to the inner leaflet of the cell membrane, are expressed in the outer portion of the lipid bilayer during apoptosis. They are recognized by Gas6 gamma-carboxyglutamic acid residues. TAM receptors present on macrophages bind their ligand, leading to phagocytosis of the apoptotic cell and inducing furthermore an anti-inflammatory state in macrophages(35).

Gas6 could thus have a protective role in sepsis, functioning as a counter regulator of the “cytokine storm”. However, as previously stated, only a minor part of fatal outcomes is linked to this phenomena, the majority of them being linked to the immunosuppressive state that occurs in sepsis. This later is due to direct effectors cells apoptosis, mainly lymphocyte cells, induced notably by cytokines, but also to the anti-inflammatory programme in macrophage following apoptotic cells phagocytosis(7). The immunomodulatory effect of Gas6 could then be double-edged: it decreased pro-inflammatory cytokine production and thus participates in immunosuppression.

An other fact that enhances complexity when trying to understand the role of Gas6 during sepsis is that it acts as an activation amplifier on endothelium. It induces pro-inflammatory cytokine secretion by endothelial cells. Furthermore, Gas6 promotes expression of leukocytes adhesion molecules, such as VCAM and ICAM by endothelial cells.

Thus, Gas6 has opposite effect on inflammation, reducing and augmenting macrophages and endothelium participation, respectively. The higher Gas6 level in dead patients could be interpreted as a result of an higher inflammatory state, in an attempt to regulates it, or as a mediator of endothelial activation, immunoparalysis and sepsis pathophysiology.

The soluble receptor sAxl is also more elevated in non-survivors than in survivor. As demonstrated by Ekman et al., sAxl is present in human plasma and can bind Gas6, possibly inhibiting its effects(34). However, according to our results, it correlates positively with Gas6 and is more elevated in non-survivors than in survivors. As it is the free portion

of Gas6 that seems biologically active, the question is if the circulating Gas6 that is measured has a physiological effect. Whatever, even if inactive when bound to sAxl, circulating Gas6 concentration might be the reflect of its effects on local cells were it is secreted.

In conclusion, our results demonstrate that Gas6 plasma level at admission in patients with severe sepsis or septic shock were higher in non-survivors than in survivors and this remains constant during ICU stay. Gas6 was a better mortality predictor than the other tested markers in patients with septic shock and might be a useful tool to predict mortality in those patients. Thus, Gas6 hold promise as an early sepsis marker. However, its precise pathophysiological role in sepsis remains to be elucidated. Our observation should be further investigated in large prospective clinical trials.

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Figures and tables

Figure legends

Figure 1: Acting through the TAM receptor family, Gas6 promotes interaction between endothelium and leukocytes by inducing expression of adhesion molecules such as VCAM-1 and ICAM-1 (1) and induces endothelial cells cytokine secretion, notably IL-1beta and IL-6(2). Gas6 interacts with phosphatydilserine expressed on apoptotic cells and induces phagocytosis by monocytes/macrophages(3). Finally, it downregulates pro-inflammatory production such as TNF-alpha, IL-6 and IL-1 in monocytes/macrophages(4).

Figure 2: Gas6 plasma levels at admission in survivors and non-survivors (in % of normal plasma). **p=0.0027.

Figure 3: Gas6 levels evolution during ICU stay based on a linear mixed model. Round and cross represent the measures in deceased person and survivors, respectively.

Figure 4: ROC curve of Gas6 level at admission in patients with septic shock.

Figure 5: Survival curves of patients with septic shock (n=67), stratified by Gas6 measurement at admission. ***p=0.0009

Figure 6: sAxl plasma levels at admission in survivors and non-survivors (in ng/mL). *p= 0.01

Table 1. Characteristics of study population

Variables	n (%)	median (P25-P75)
Total patients	94	
Age (yrs)		65 (49.5-74)
Gender		
Male	57 (60.64)	
Female	37 (39.36)	
Outcome		
Survivors	73 (77.66)	
Non-survivors	21 (22.34)	
Cause		
Septic shock	66 (70.21)	
Severe sepsis	28 (29.79)	
Principal diagnosis leading to ICU admission		
Pneumonia	31 (33.0)	
Peritonitis	25 (26.6)	
Fasciitis	12 (12.8)	
Cholangitis	5 (5.3)	
Pyelonephritis	5 (5.3)	
Others	16 (17.0)	
Pathogen		
E. Coli	22 (23.4)	
S. pneumoniae	18 (19.1)	
None	13 (13.8)	
S. pyogenes	10 (10.6)	
Pseudomonas aeruginosa	6 (6.4)	
S. aureus	6 (6.4)	
Others	19 (20.3)	
Gravity scores		
APACHE II		27.5 (23.3-37)
SOFA		11 (9-13)
CRP (mg/L)		231.5 (130.8-315)
PCT (µg/L)		22.3 (5.2-53.3)

ICU = Intensive Care Unit ; APACHE II = Acute Physiology and Chronic Health Evaluation II score ; SOFA = Sequential Organ Failure Assessment ; CRP = C-reactive protein ; PCT= Procalcitonin

Table 2. Correlations and ROC curves area

a)

Marker	rho	p
PCT	0.18	0.09
CRP	-0.04	0.72
SOFA	0.10	0.36
APACHE2	0.04	0.68
* TNF-alpha	0.20	0.06
* IL-6	0.24	0.02
* IL-8	0.32	0.00
* IL-10	0.36	0.00
* IL-1 beta	-0.05	0.63

b)

Marker	ROC Area	SE	95% CI	
Gas6	0.72	0.07	0.57	0.85
PCT	0.44	0.08	0.28	0.59
CRP	0.42	0.06	0.29	0.54
TNF-alpha	0.43	0.09	0.40	0.74
IL-6	0.61	0.08	0.50	0.82
IL-8	0.60	0.09	0.47	0.81
IL-10	0.62	0.09	0.48	0.84
IL-1beta	0.44	0.09	0.30	0.63
SOFA	0.51	0.08	0.36	0.66
APACHE2	0.57	0.07	0.43	0.71

* n=90

a) Correlations between Gas6 and different markers and scores.
b) ROC curve area of the different markers and scores.

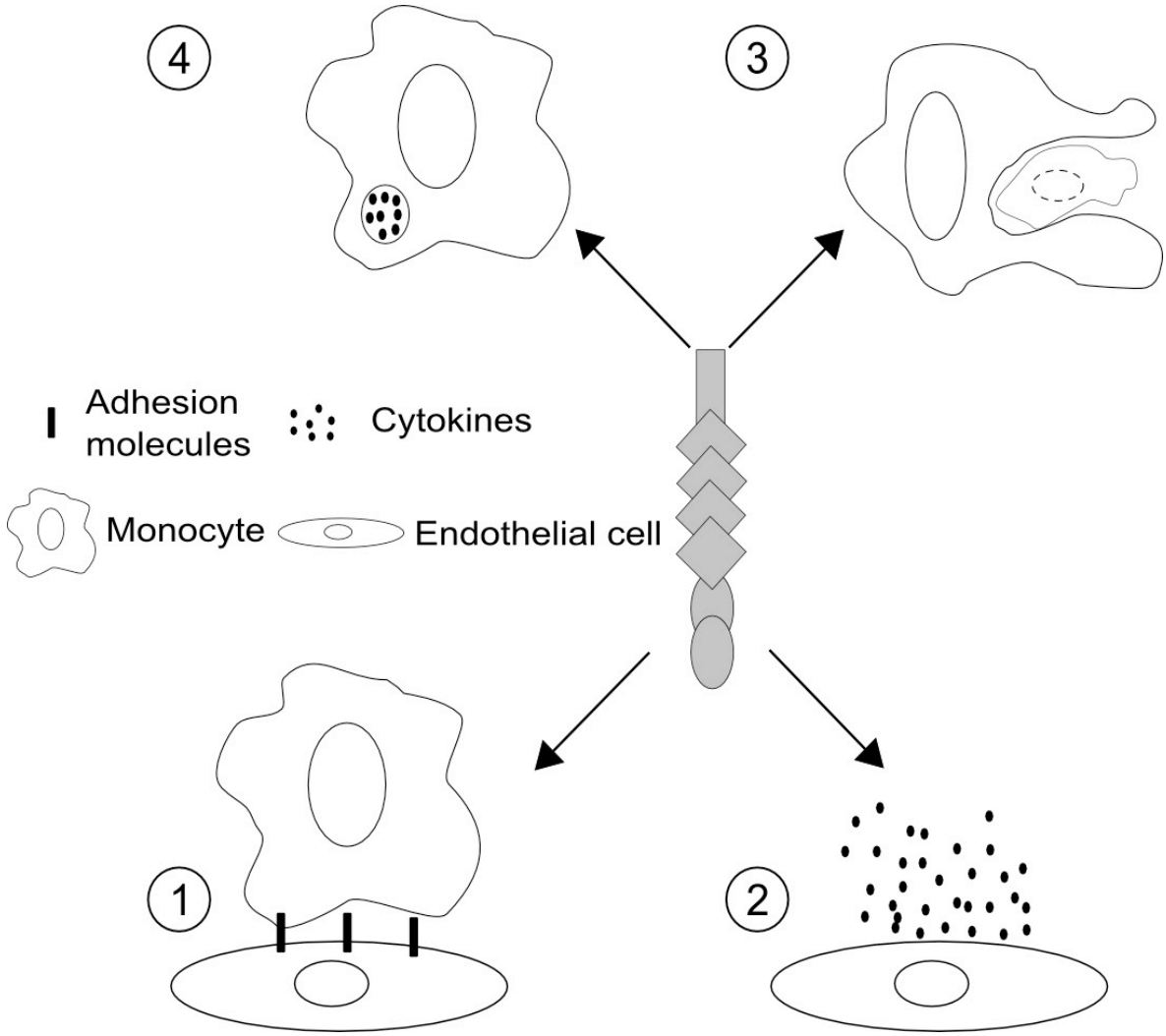

4
3
Adhesion
molecules
Cytokines
Monocyte
Endothelial cell
1
2

Figure 1

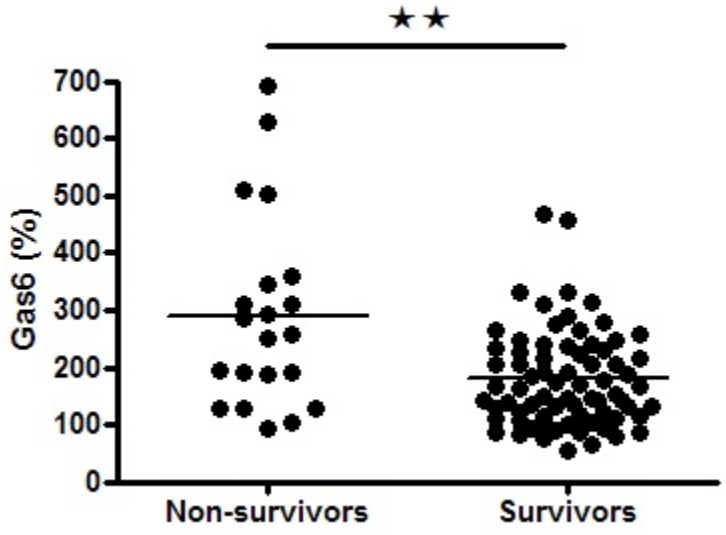


Figure 2

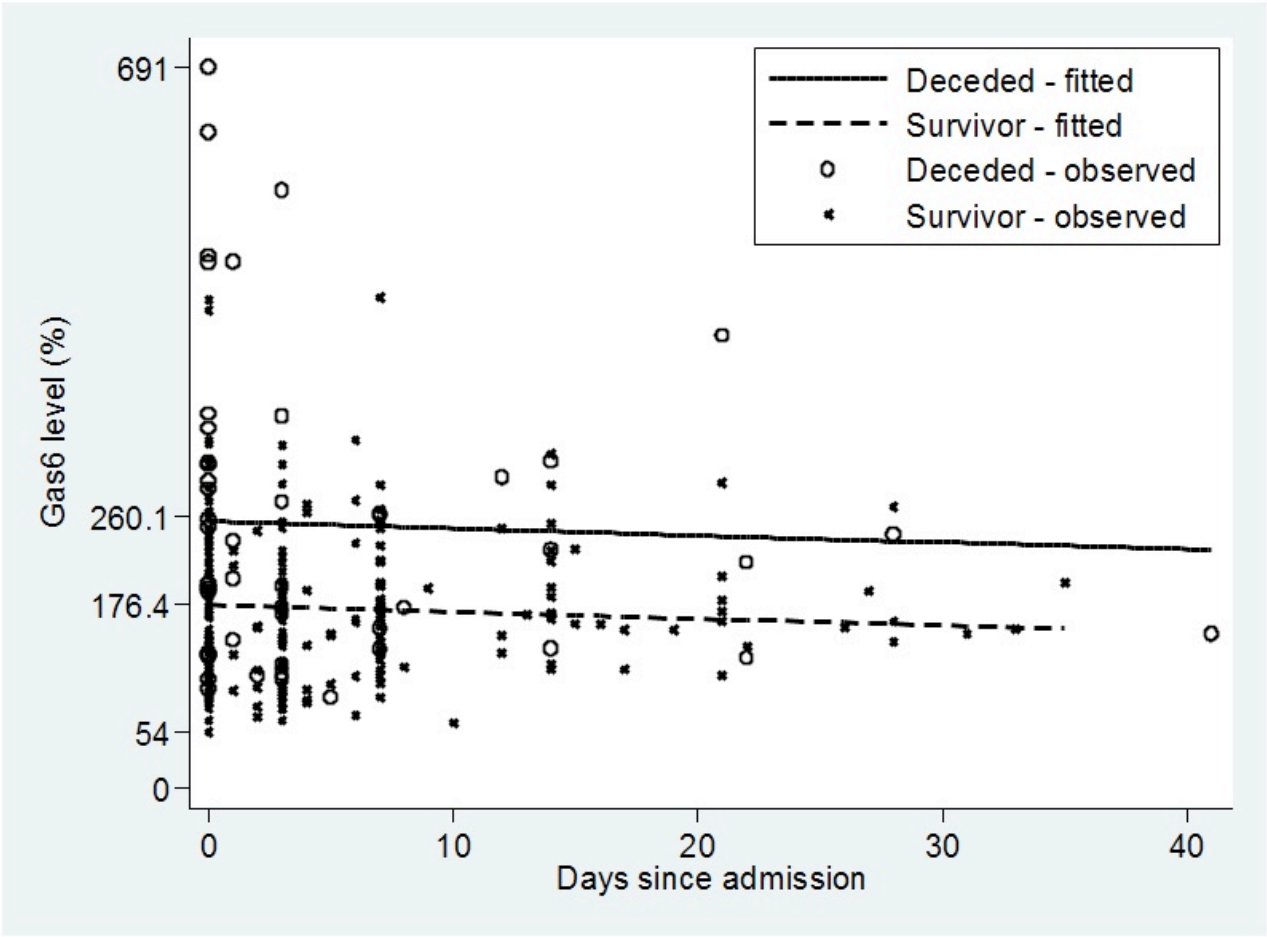
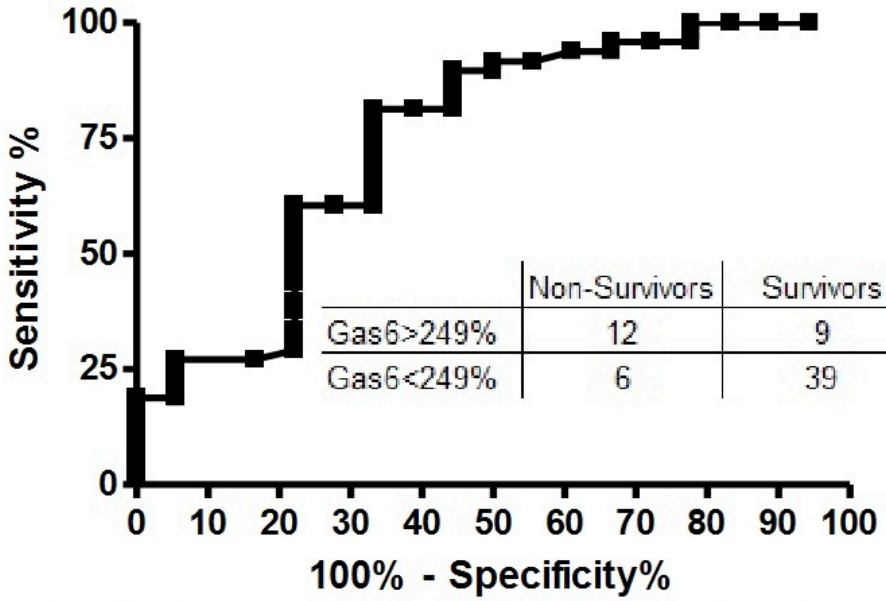

691
260.1
176.4
54
0
Gas6 level (%)
0
10
20
30
40
Days since admission
Deceded - fitted
Survivor - fitted
Deceded - observed
Survivor - observed

Figure 3



	Non-Survivors	Survivors	
Gas6>249%	12	9	PPV=57%
Gas6<249%	6	39	NPV=87%

Cutoff	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
< 249.0	81.25	67.37% to 91.05%	66.67	40.99% to 86.66%	2.44

Figure 4

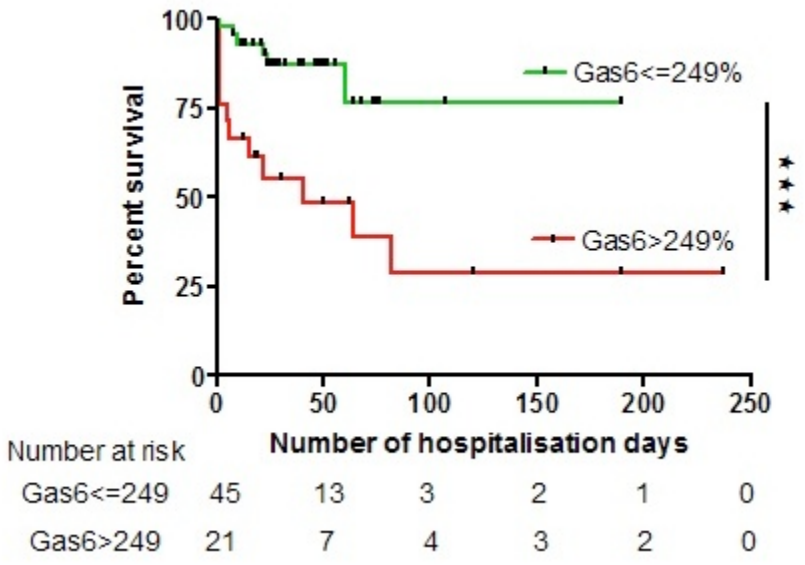

100
75
50
25
0
Percent survival
Gas6<=249%
Gas6>249%
***
0
50
100
150
200
250
Number of hospitalisation days
Number at risk
Gas6<=249 45 13 3 2 1 0
Gas6>249 21 7 4 3 2 0

Figure 5

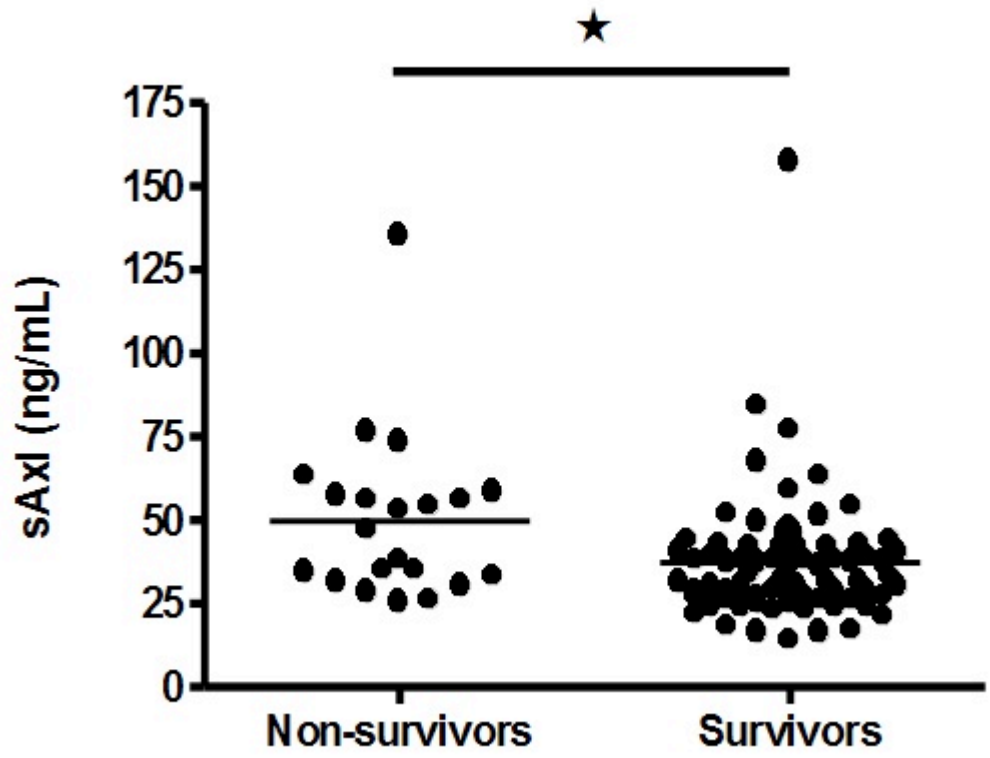


Figure 6

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